

Immunosenescence shapes the inflammatory response to viral infection in vaccinated and unvaccinated patients

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Abstract

A key feature of immunosenescence is chronic low-grade inflammation, or inflammaging, which disrupts antigen-specific signaling and limits the magnitude, quality, and durability of vaccine-induced immunity. The study aimed to characterize the inflammatory response to viral infection through a comprehensive assessment of immune cells and cytokines in unvaccinated (NVP) and vaccinated patients (VP), and to investigate the impact of immunosenescence on vaccination effectiveness. The study included 79 patients aged 61.1 ± 16.7 years with newly diagnosed COVID-19 and 41 healthy, unvaccinated individuals aged 71.4 ± 4.5 years as healthy comparators (HC). Changes in neutrophil and lymphocyte counts resulted in the pan-immune-inflammation value (PIV) increase in infected patients (NVP 909 ± 1341 , VP 1774 ± 2645) compared to HC (233 ± 204). Area under the curve (AUC) values derived from a multivariable logistic regression model for PIV and cytokines provided excellent discrimination between healthy individuals and infected patients: PIV + IL-1 β (AUC = 0.909), PIV + IL-1Ra (AUC = 0.877), PIV + IL-6 (AUC = 0.898), and PIV + IL-8 (AUC = 1.000), PIV + IL-10 (AUC = 0.839) and PIV + TNF α (AUC = 0.998). Patients aged ≥ 65 years had significantly higher levels of IL-1 β ($p = 0.036$), IL-1Ra ($p = 0.025$), IL-6 ($p = 0.026$), IL-8 ($p = 0.014$), IL-10 ($p = 0.005$), TNF α ($p = 0.071$) and INF γ ($p = 0.040$) than those aged < 65 , regardless of vaccination status. Overall, these findings reveal a complex interplay within the immune system in response to viral infection and vaccination. Age strongly shapes the immune response following vaccination by altering the numbers, distribution, and functional states of neutrophils, thereby complicating the course of COVID-19. The predictive value of neutrophil-related cytokines increased when PIV was included in inflammatory profiling.

1. Introduction

Population aging poses a crucial challenge to modern vaccinology, as declining vaccine efficacy reflects not antigen failure but age-associated remodeling of the immune system, characterized by the accumulation of dysfunctional, senescent immune cells [1–3]. Central to immunosenescence is chronic low-grade inflammation, or inflammaging, which disrupts antigen-specific signaling and limits the magnitude, quality, and durability of vaccine-induced immunity [4]. Numerous studies on the capacity of influenza vaccines show age-related differences in immune protection, with efficacy ranging from 70% to 90% in young people but decreasing to 30–50% in people over 65 years of age [5]. Influenza mRNA vaccines elicit substantially higher seroconversion rates than inactivated vaccines, suggesting a promising strategy to enhance immune protection in this population, which is at elevated risk of infection [6]. However, these findings should be interpreted with caution given the small number of studies available.

The phenotype of senescent immune cells changes substantially, resulting in the development of the profile of secretory factors called senescence-associated secretory phenotype (SASP). SASP is manifested by the release of a large number of pro-inflammatory factors, such as interleukins (IL) IL-1 β , IL-2, IL-6, IL-8, IL-12, IL-15, IL-18, IL-22, IL-23, tumor necrosis factor α (TNF α) and interferon γ (IFN γ), together with reduced anti-inflammatory cytokines such as IL-1Ra, IL-4, IL-10 and IL-13 [7, 8]. Several

SASP components, including IL-1 β , IL-6, IL-8 and TNF α , are all potential targets for pharmacological inhibition. Some of them are already used in clinical practice and may be exploited to mitigate the deleterious effects of SASP in the relevant clinical context [9]. Interestingly, these cytokines are the crucial inflammatory mediators that adjust both the cellular and humoral immune responses to infection through complex ligand-receptor interaction networks. In turn, uncontrolled production of pro-inflammatory cytokines during the COVID-19 pandemic has been documented as a hallmark of inflammaging linked to increased disease severity and mortality [10]. Older adults generally exhibit lower pro-inflammatory cytokine responses after inactivated virus COVID-19 vaccines compared to younger individuals, which results in a delayed or lower antibody production [11]. Similarly, humoral responses to COVID-19 mRNA vaccines are significantly weaker in older adults, and antibody-mediated activity in plasma decline universally over time. Consequently, older adults may remain at elevated risk of infection despite vaccination [12–14]. Therefore, further research on inflammaging, its impact on vaccine response, and its immunological mechanisms is needed to provide scientific insights to optimize vaccines for geriatric population.

Limited information is available on monitoring cytokine response as a marker of COVID-19-related inflammation following vaccination with mRNA vaccines in older population [12, 14–16]. Few studies have shown that mRNA vaccination in older individuals results in reduced and delayed cellular cytokine response and higher resting levels of baseline inflammaging. While older adults still generate robust antibody levels, the overall magnitude of the T-cell-mediated cytokine response is frequently weaker compared to younger individuals [12, 14]. mRNA vaccines are delivered using lipid nanoparticles which can act as mild adjuvants and trigger an early and transient release of pro-inflammatory cytokines in the general population [17]. This response involves mainly IL-1, IL-6, and TNF α which are essential for recruiting immune cells and building long-lasting immunity [18]. The very same cytokines are also responsible for the primary drivers of common, transient vaccine side effects including localized swelling, fever, and fatigue [19]. In a mouse model, inhibition of IL-6 effectively blunted the adverse reactions while maintaining the vaccine efficacy. These data provide basic information for understanding adverse reactions in humans and may contribute to the development of mRNA vaccines with improved tolerability [19]. According to Collier et al. [12], serum neutralization and levels of binding IgG or IgA after the first vaccine dose were lower in older individuals, with a marked drop among participants over 80 years old. However, following the second dose, neutralization against variants of concern was detectable regardless of age. The frequency of SARS-CoV-2 spike-specific memory B cells was higher in elderly responders (whose serum showed neutralization activity) than in non-responders after the first dose. Elderly participants also demonstrated a clear reduction in somatic hypermutation of class-switched cells. Furthermore, production of IFN γ and IL-2 by SARS-CoV-2 spike-specific T cells was lower in older participants, and both cytokines were secreted primarily by CD4 T cells [12]. COVID-19 remains a recurring public health issue, and the elderly continue to face a disproportionately high risk of severe illness, hospitalization, and reinfection. The specific measures to monitor cytokine response and to boost vaccine effectivity in this group are warranted, particularly among non-responders after the first vaccine dose. Addressing this knowledge gap is essential for developing optimal vaccination strategies

to enhance immunity in this age group. Therefore, this study was designed to: 1) determine the inflammatory response to SARS-CoV-2 infection through comprehensive assessment of immune cells and cytokines, 2) establish the relationship between inflammaging and COVID-19 severity in unvaccinated and vaccinated patients, 3) explain whether inflammaging may serve as a predictor of impaired vaccine response.

2. Material and methods

2.1. Study population.

The study included seventy-nine patients aged 61.1 ± 16.7 years with newly diagnosed COVID-19 who were admitted to Intensive Care Unit at the University Hospital in Zielona Gora (Poland) from December 2021 to June 2022 (Table 1). Patients underwent diagnostic procedures and therapy according to the standards of the National Health Fund (Poland), and in accordance with our previously described therapeutic approach [20]. Patients were excluded if they did not exhibit clinical symptoms consistent with COVID-19 [21], had no confirmed positive RT-PCR test for COVID-19, or lacked complete clinical data such as lung computed tomography (CT) scans. Among patients with COVID-19, 39 individuals aged 60.8 ± 15.9 years were unvaccinated (NVP, female, $n = 19$, male, $n = 20$) whereas 40 individuals aged 70.9 ± 12.4 years were vaccinated (VP, female, $n = 19$, male, $n = 21$). Vaccinated patients had received two doses of either mRNA vaccine Pfizer-BioNTech ($n = 37$) or viral vector vaccine AstraZeneca ($n = 3$). The interval between the first and the second doses was 37.3 ± 16.6 days (median 34.0 days, IQR 24–38). The COVID-19 groups (NVP and VP) were compared to 41 healthy individuals (healthy unvaccinated comparators, HC) aged 71.4 ± 4.5 years (female, $n = 21$, male, $n = 20$) who were recruited at the University of the Third Age (Zielona Gora Poland). The current health status of HC was assessed on the basis of medical records at a routine follow-up visit to a primary care physician. On the basis of the medical interview, the following exclusion criteria were applied for HC: obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$), systemic autoimmune disorders under immunosuppressive therapy, active malignancies, active acute infections, and other immune-mediated disorders. The medications taken by the HC included antihypertensive (83%) and hypolipidemic (11%) drugs as well as anticoagulants including antiplatelet agents (15%). The study was approved by the institutional review board at University of Zielona Gora (No. 21/157/2021). The study adhered to the principles and standards outlined in the Declaration of Helsinki and the Guidelines for Good Clinical Practice. Written informed consent was obtained from all participants before enrollment.

2.2 Clinical analysis.

COVID-19 patients were stratified according to the clinical risk score by Liang et al. [22]. General ward admission criteria included characteristic radiographic findings and/or shortness of breath defined as tachypnea and/or low oxygen pulse in the absence of alternative diagnosis. The criteria for Intensive Care Unit admission included low oxygen pulse despite supplementary oxygen delivered via a non-rebreather mask, sepsis according to the Sequential Organ Failure Assessment (SOFA) criteria and/or

the need for mechanical ventilation, a vasopressor-requiring shock, and unexplained confusion. The diagnosis of SARS-CoV-2 infection was confirmed by RT-PCR of nasopharyngeal swab samples using the MagNA Pure 24 or MaelStrom with a real-time thermal cycler Cobas 480 (Roche Diagnostics, USA). All testing was performed in the clinical laboratory of the University Hospital in Zielona Gora. All patients received standard supportive care, including low-molecular-weight heparin, statins, and supplementary oxygen as required. Patients with severe COVID-19 and oxygen saturation of $\leq 90\%$ additionally received corticosteroids while critically ill patients received tocilizumab (Table 1).

Table 1
Characteristics and treatment of COVID-19 patients
(*n* = 79).

Sex, n (%)	
Female	38 (48.1%)
Male	41 (51.9%)
Age, n (%)	
< 65 years	30 (38.0%)
≥65 years	49 (62.0%)
Comorbidities, n (%)	
Arterial hypertension	33 (41.8%)
Atrial fibrillation	6 (7.6%)
Coronary heart disease	10 (7.9%)
Heart failure	5 (6.3%)
Chronic kidney disease	5 (6.3%)
Diabetes	14 (17.7%)
Cancer	5 (6.3%)
Lung diseases*	8 (10.1%)
Obesity (BMI ≥30 kg/m ²)	21 (26.6%)
CT lung involvement, n (%)	
< 25%	33 (41.8%)
25–50%	19 (24.0%)
50–75%	19 (24.0%)
> 75%	8 (10.1%)
Therapy, n (%)	
Anticoagulants and antithrombotics	7 (8.8%)
Corticosteroids	79 (100%)
Oxygen therapy	79 (100%)
Tocilizumab	3 (3.8%)

* Bronchial asthma, chronic obstructive pulmonary disease.

2.3 Blood sampling.

Fasting blood samples were collected from the median cubital vein in the morning between 7:00 and 9:00 using S-Monovette tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). The whole blood samples were placed into specimen tubes containing EDTA-K3 and were immediately analyzed. For the other biochemical analyses blood samples were centrifuged at 3000 rpm for 10 min, and aliquots of plasma were stored at -80°C.

2.4 White blood cell count-derived inflammatory indices.

Hematological parameters including total white blood cell count (WBC), platelet count and WBC differential count were determined using Sysmex XN-1000 (Sysmex Europe GmbH, Norderstedt, Germany). The pan-immune-inflammation value (PIV) was calculated according to the following formula: $PIV = [\text{neutrophil count (} 10^3/\mu\text{L)} \times \text{platelet count (} 10^3/\mu\text{L)} \times \text{monocyte count (} 10^3/\mu\text{L)}] / \text{lymphocyte count (} 10^3/\mu\text{L)}$ proposed by Fucà et al. [23].

2.5 Inflammatory variables.

Plasma concentrations of interleukin 1 β (IL-1 β), interleukin 1 receptor antagonist (IL-1Ra), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), TNF α and IFN γ were determined using ELISA kits from SunRed Biotechnology Company (Shanghai, China) with detection limits of 28.384 pg/mL, 28.125 pg/mL, 1.867 pg/mL, 1.869 pg/mL, 1.142 pg/mL, 2.782 pg/mL and 1.706 pg/mL, respectively. The mean intra-assay coefficients of variation (intra-assay CV) for the used enzyme immunoassay tests (ELISA) were < 8%. All samples were analyzed in duplicate in a single assay to avoid inter-assay variability.

2.6 Statistical analysis.

Statistical analyses were performed using R 4.5.1 software [24]. Continuous variables were reported as mean values $\pm$ standard deviation (SD) and median (Me). Statistical differences among groups were assessed using the Kruskal-Wallis rank-sum test, followed by Dunn's post hoc test with Holm adjustment for multiple comparisons. The predictive values of variables were evaluated by measuring the area under the receiver operating characteristic (ROC) curve with corresponding 95% confidence interval (CI). The optimal threshold value for clinical stratification (cut-off value) was determined by calculating the Youden index. Spearman's rank correlation coefficients were calculated to assess the relationships between inflammatory markers, and their statistical significance was evaluated using the corresponding Spearman's rank correlation test. Statistical significance was set at 0.05.

3. Results

3.1 Study population.

According to the standard benchmark used to define an aging immune system [25], 49 patients with COVID-19 were aged $\geq$ 65 years, and 60% of them were vaccinated. Among VP, the time from the last

vaccine dose to the onset of COVID-19 symptoms was 179 ± 62 days (median 184.0, IQR 143-222), while the time from symptom onset to hospitalization was 6.2 ± 5.4 days (median 5.0, IQR 2.3-8.0). Among NVP, the time from symptom onset to hospitalization was 6.0 ± 4.1 days (median 7.0, IQR 3.0-7.0). CT showed lung involvement $\geq 50\%$ in 27 patients (Table 1), which confirmed the severity of COVID-19. The vaccination reduced the degree of lung involvement and mortality. Specifically, 11 vaccinated patients had $\geq 50\%$ lung involvement and 5 died, whereas 16 unvaccinated patients had lung involvement $\geq 50\%$ and 9 died. CT results were not associated with patient age i.e., patients with lung involvement $< 50\%$ were aged 61.8 ± 16.9 years, and patients with $\geq 50\%$ lung involvement were aged 61.4 ± 14.7 years. The burden of comorbidities and/or obesity and the extent of lung involvement had no effect on immunoinflammatory status in patients. However, age was associated with immunoinflammatory status when patients were stratified according to the standard age threshold used to define an aging immune system (≥ 65 years) [25]. Patients aged ≥ 65 years demonstrated higher levels of both pro- and anti-inflammatory cytokines IL-1b, IL-1Ra, IL-6, IL-8, IL-10 and TNFa compared to patients aged < 65 years. This clearly shows that immunosenescence affects the immune response to viral infection.

3.2 White blood cell count-derived inflammatory indices.

The Kruskal-Wallis test revealed statistically significant differences in the distributions of WBC, neutrophils, lymphocytes, platelets, PIV and CRP ($p < 0.001$) among the HC, NVP and VP groups, as well as in monocyte counts ($p = 0.025$). WBCs, platelets, and differential white blood cell counts fell within the reference range in HC and were significantly lower than those observed in patients with COVID-19. The exception was the lymphocyte count, which was significantly higher in healthy individuals (Table 2). WBC counts exceeded upper reference limit in 30% of COVID-19 patients (range, 10.39 - 30.40). Changes in neutrophil and lymphocyte counts were reflected in increased PIV values. PIV was significantly ($p < 0.0001$) higher in NVP (909 ± 1341 , median 381) and VP (1774 ± 2645 , median 825) compared to HC (233 ± 204 , median 194). In COVID-19 patients PIV was clearly more dispersed than in healthy individuals. The difference was particularly pronounced in men (1573 ± 2757) compared to women (1115 ± 1206) indicating sex-related differences in inflammatory response during viral infection. A similar distribution of values was observed for C-reactive protein (CRP) levels, which were higher in men (117 ± 110 mg/L) than in women (79 ± 74 mg/L) with COVID-19. Both NVP and VP were simultaneously characterized by elevated PIV levels and increased concentrations of pro-inflammatory cytokines, especially those responsible for neutrophil activation i.e., IL-1b, IL-6 and TNFa (Table 3). Age significantly influenced the PIV response to vaccination status. Among unvaccinated patients, PIV was higher in those aged ≥ 65 years (Median 506) compared with those aged < 65 years (median 315). By contrast, in the vaccinated group the immune response pattern was reversed i.e., PIV was lower in older (median 658) than in younger (median 1090) individuals. For PIV, the AUC was 0.839 (95% CI 0.767-0.912, sensitivity 0.848, specificity 0.757, $p < 0.0001$) with a cut-off value of 257, indicating its strong discriminatory potential.

Table 2. White blood cell counts and C-reactive protein.

Variables	Reference values	HC <i>n</i> =41	NVP <i>n</i> =39	VP <i>n</i> =40	<i>p</i> level		
		Mean ± SD (Me)	Mean ± SD (Me)	Mean ± SD (Me)	HC vs. NVP	HC vs. VP	NVP vs. VP
WBC x10 ³ /μL	4.0–10.2	6.02 ± 1.63 (5.72)	7.65 ± 4.04 (6.74)	10.3 ± 5.65 (9.2)	0.092	<0.001	0.005
Neutrophils x10 ³ /μL	2.0–6.9	3.37 ± 1.30 (3.09)	6.09 ± 3.8 (5.04)	8.22 ± 5.33 (6.56)	<0.001	<0.001	0.022
Lymphocytes x10 ³ /μL	0.6–3.4	1.92 ± 0.69 (1.78)	0.96 ± 0.41 (0.89)	1.27 ± 0.71 (1.11)	<0.001	<0.001	0.042
Monocytes x10 ³ /μL	0.00–0.90	0.50 ± 0.12 (0.53)	0.52 ± 0.29 (0.46)	0.64 ± 0.27 (0.59)	0.461	0.110	0.026
Platelets x10 ³ /μL	140–420	226 ± 62 (215)	191.26 ± 105.02 (166)	265.41 ± 128.88 (251.9)	0.012	0.389	0.001
CRP mg/L	0.00-5.00	3.09 ± 2.23 (2.91)	92.73 ± 78.76 (63.60)	103.37 ± 112.27 (69.75)	<0.001	<0.001	0.878

Abbreviations: HC healthy unvaccinated comparator, NVP unvaccinated patients with COVID-19, VP vaccinated patients with COVID-19, WBC white blood cells, CRP C-reactive protein.

3.3 Inflammatory variables.

Elevated levels of IL-1b, IL-6, IL-8 and TNFa were observed, which indicates that inflammation is a critical component of COVID-19 progression (Table 3). The Kruskal-Wallis test revealed statistically significant differences in the distributions of IL-1β, IL-1Ra, IL-6, IL-8, and TNFa among the HC, NVP and VP groups (*p*<0.001). The most considerable changes were recorded for pro-inflammatory cytokines IL-8 and TNFa, which were approximately 18-fold higher in COVID-19 patients than in healthy individuals (*p*<0.001). In contrast, no significant differences were observed for IL-10 and IFNγ in study population (Table 3). IL-1b, IL-1Ra, IL-6 and TNFa demonstrated particularly high levels in VP group, which suggests that vaccination may have increased the overall inflammatory burden. The heatmap illustrating the relationships between inflammatory variables showed no correlation in the healthy comparator group, whereas, multiple correlations were observed in COVID-19 patients (Figure 1). A particularly strong correlation was detected between IL-8 and IL-10 (rho=0.79) in both NVP and VP. According to a standard benchmark

used to define an aging immune system [25], patients aged ≥ 65 years demonstrated significantly higher levels of IL-1b ($p=0.036$), IL-1Ra ($p=0.025$), IL-6 ($p=0.026$), IL-8 ($p=0.014$), IL-10 ($p=0.005$), TNFa ($p=0.071$) and INFg ($p=0.040$) compared to younger ones <65 years of age. Patients aged ≥ 65 years also demonstrated a higher IL-6 /INFg ratio (2.50 ± 4.29 pg/mL, median 1.25) compared to patients aged <65 years (0.92 ± 0.99 pg/mL, median 0.59), which indicates an ineffective antiviral response at advanced age. No differences in cytokine response depending on vaccination status were observed among either older or younger patients. In univariate ROC curve analysis, inflammatory markers exhibited poor discriminative ability, with AUC values ranging from approximately 0.50 to 0.67. The 95% confidence intervals included 0.5, confirming that individual cytokines alone, evaluated without taking into account other immune or clinical factors, did not reliably differentiate healthy individuals from patients with COVID-19. However, the predictive values increased when PIV was incorporated into the logistic regression models for cytokines (Figure 2). A multivariable logistic regression model combining PIV with individual cytokines demonstrated excellent discrimination between healthy individuals and patients with COVID-19: PIV + IL-1b (AUC=0.909), PIV + IL-1Ra (AUC=0.877), PIV + IL-6 (AUC=0.898), and PIV + IL-8 (AUC=1.000), PIV + IL-10 (AUC=0.839) and PIV + TNFa (AUC=0.998). The high AUC values indicate that these classifiers have excellent discriminatory performance and may provide useful tools for differentiating patients with COVID-19 from healthy individuals based on comprehensive inflammatory profiling.

Table 3. Inflammatory variables.

Variables	HC <i>n</i> =41	NVP <i>n</i> =39	VP <i>n</i> =40	<i>p</i> level		
	Mean ± SD (Me)	Mean ± SD (Me)	Mean ± SD (Me)	HC vs. PNV	HC vs. PV	PNV vs. PV
IL-1b pg/mL	1077 ± 531 (883)	1917 ± 1130 (1720)	2521 ± 1425 (2135)	< 0.001	<0.001	0.025
IL-1Ra pg/mL	802 ± 264 (847)	1177 ± 730 (922)	1436 ± 598 (1308)	0.015	<0.001	0.008
IL-6 pg/mL	46.34 ± 15.18 (47.25)	94.77 ± 88.37 (72.22)	232.09 ± 449.22 (113.00)	<0.001	<0.001	0.002
IL-8 pg/mL	8.97 ± 4.31 (8.53)	154.57 ± 145.91 (108.93)	147.50 ± 90.12 (130.09)	<0.001	<0.001	0.722
IL-10 pg/mL	48.05 ± 8.23 (51.06)	60.77 ± 60.36 (46.83)	62.58 ± 48.01 (55.06)	1.000	1.000	1.000
TNFa pg/mL	63.08 ± 26.07 (65.10)	507.57 ± 394.89 (348.31)	808.80 ± 728.38 (516.79)	<0.001	<0.001	0.227
IFNγ pg/mL	128.88 ± 55.63 (123.06)	106.8 ± 65.53 (84.72)	130.85 ± 73.03 (98.09)	0.077	0.662	0.148

Abbreviations: HC healthy unvaccinated comparator, NVP unvaccinated patients with COVID-19, VP vaccinated patients with COVID-19, IL-1β interleukin 1β, IL-1Ra interleukin-1 receptor antagonist, IL-6 interleukin 6, IL-8 interleukin 8, IL-10 interleukin 10, TNFa tumor necrosis factor a, IFNγ interferon g.

4. Discussion

4.1

The persistent presence of senescence-associated secretory phenotype creates a suppressive microenvironment that prevents dendritic cells from properly processing and presenting vaccine antigens to the adaptive immune system. Moreover, SASP factors overstimulate the innate immune system, leading to T-cell and B-cell exhaustion and reducing the production of neutralizing antibodies following a vaccination [14, 26]. A critical aspect of cellular immunity is the synthesis and regulation of cytokines, which play a central role in orchestrating immune responses. Key cytokines involved in the immune response to vaccination include IL-1b, IL-1Ra, TNFa, IL-6, IL-8, IL-10, and IFNγ [27]. TNFa is vital for antiviral defense and the immune cells activation, while IFNγ enhances local antiviral responses and stimulates immune activity [28]. IL-6 is a pleiotropic cytokine that functions as both a pro-inflammatory and anti-inflammatory mediator during viral infections, and its dual nature depends on the concentration of the cytokine [29]. IL-1Ra and IL-10 act as immunosuppressants, preventing excessive immune reactions [30]. IL-8 is released in large amounts during viral infections and recruits and activates

neutrophils at sites of acute inflammation. By binding to CXCR1 and CXCR2 receptors on the neutrophil surface, IL-8 induces rapid chemotaxis, adhesion, and transendothelial migration into tissues [31]. The regulation of these cytokines during vaccination is therefore crucial, as it directly influences the magnitude and quality of vaccine-induced protection [11].

4.2

In our study, immune cell recruitment was particularly evident for neutrophils, resulting in a 4-fold increase in PIV in patients with COVID-19 compared to healthy individuals (AUC=0.839, sensitivity 0.848, specificity 0.757, $p<0.0001$). Reduced lymphocyte counts amplified the increase in PIV values. Patients with COVID-19 were also characterized by elevated levels of pro-inflammatory cytokines responsible for recruitment and activation of neutrophils, including IL-1b, IL-6, IL-8 and TNF α . According to WHO summary statistics on COVID-19, approximately 19% of patients had elevated serum levels of proinflammatory cytokines and various immune cells [32]. In our study, IL-1b and IL-6 concentrations were approximately twofold higher in patients with COVID-19 compared to healthy individuals. Elevated IL-6 levels have been consistently reported in patients with COVID-19 [33-35], suggesting that IL-6 might have potential as a disease severity predictor. However, in our study IL-8 showed the greatest increase in response to SARS-CoV-2 infection (NPV: median 108.93, VP: median 130.09) compared to healthy individuals (Median 8.53), which identifies IL-8 as the strongest candidate biomarker for predicting disease severity and prognosis in COVID-19 [36-38]. Multivariate logistic models combining PIV with cytokines, including IL-1b, IL-6, IL-8 and TNF α , demonstrated very high diagnostic potential in differentiating between healthy and infected individuals (AUC 0.898-1.000). The release of endothelial cytokines IL-1 α , IL-6, IL-8, and TNF α do not only contribute to peripheral neutrophilia and lymphopenia, but also to increased capillary permeability, platelet activation, enhanced coagulation, reduced fibrinolysis, and overactivation of the immune and hemostatic systems [39-41]. Together, these pathomechanisms provide the rationale for integrating lymphocytes, neutrophils, monocytes, and platelet counts with cytokine measurements to estimate disease risk and predict COVID-19 outcomes.

4.3

The compensatory anti-inflammatory mechanism was represented by IL-1Ra and IL-10. IL-1Ra highly correlated with pro-inflammatory cytokines IL-1 β , IL-6, IL-8, and TNF α ($\rho>0.6$, $p<0.001$) particularly in unvaccinated patients (Figure 1 NVP), whereas IL-10 levels did not differ significantly among the study groups. High IL-10 level was observed only in individual severe cases in the unvaccinated group. Insufficient anti-inflammatory activity of this cytokine likely contributes to the overall impairment of the immune system, viral control, and disease severity [42]. The role of IL-1Ra in the immune response may vary depending on the severity of SARS-CoV-2 infection. In mild cases, elevated IL-1Ra levels may override the robust immune reaction to virus, whereas markedly increased IL-1Ra concentrations in severe disease indicate an overactivation of immune system, which may contribute to the switch from controlled and protective immune environment to inflammation-induced tissue damage [42]. Thus,

elevated IL-1Ra may serve as a marker of severe disease, and its synthetic form represents a potential therapeutic strategy [42].

4.4

Overall, our data confirm a cytokine milieu in patients with COVID-19 that favors neutrophil recruitment thereby sustaining an inflammatory status. Neutrophilia has been detected in the blood [43], nasopharyngeal epithelium [44] and distal lung compartments [45] during SARS-CoV-2 infection, and has been associated with a poor prognosis. In our previous study, we demonstrated significantly higher neutrophil counts ($p < 0.001$) in non-survivors ($11.62 \pm 6.30 \times 10^3/\text{mL}$) than in survivors ($8.71 \pm 4.59 \times 10^3/\text{mL}$) [43]. Neutrophilia and neutrophil overactivation can produce deleterious effects on various organs, through the induction of hyperinflammation, which is referred to as cytokine storm syndrome [46]. Various cytokines have been reported to increase in this syndrome, the most common being IL-1, IL-2, IL-2-receptor- α , IL-6, IL-8 and IL-10, IFN γ , TNF α , monocyte chemoattractant protein 1, granulocyte-macrophage colony-stimulating factor. C-reactive protein, as non-specific marker of inflammation, may also increase substantially during severe COVID-19 [47]. In our study, CRP concentrations were more than 3-fold higher in patients with COVID-19 compared to healthy individuals, and were higher in patients with lung involvement $\geq 50\%$ ($131.6 \pm 103.3 \text{ mg/L}$) compared to patients with lung involvement $< 50\%$ ($80.7 \pm 87.2 \text{ mg/L}$). Previous studies have reported that the risk of developing severe events is increased by 5% for every one-unit increase in CRP concentration in COVID-19 [48]. Thus, higher CRP levels indicate more severe disease course, associated with lung injury and a poorer prognosis.

4.5

Elevated CRP levels have been linked to the overproduction of inflammatory cytokines in patients with severe COVID-19 [49]. Monitoring CRP together with cytokines therefore allows to track disease severity and guide treatments, such as tocilizumab, for acute infectious diseases [20, 50]. CRP acts as a bidirectional mediator of inflammation. Although primarily recognized as a biomarker driven by cytokine IL-6, CRP may itself actively propagate inflammation by triggering macrophages and neutrophils to release additional IL-6 and other pro-inflammatory cytokines, including IL-1 β and TNF α [51]. Each of these cytokines reached substantially higher values in patients with COVID-19 compared to healthy individuals, especially in the vaccinated group, suggesting that vaccine increases the overall inflammatory burden. The heatmap analysis, in turn, showed numerous relationships (ρ 0.75-1.00, ρ 0.50-0.75, $p < 0.001$) between cytokines IL-1 β , IL-1Ra, IL-6, IL-8, IL-10 and TNF α in our study sample (Figure 1). In vaccinated patients, cytokine amplification was particularly evident for IL-6, with a median level of 113, representing a 1.5-fold increase compared to unvaccinated patients (median 72.22). Mink et al. [52] demonstrated that patients with high levels of inflammatory markers (CRP $> 60 \text{ mg/L}$ or IL-6 $> 100 \text{ pg/mL}$) combined with low levels of anti-SARS-CoV-2-spike antibodies were approximately 8 times more likely to die than those with low inflammatory responses and high antibody levels. A post-vaccination increase was also observed in TNF α levels (VP: median 516.79 vs. NVP: median 348.31). It is generally

accepted that an increase in TNF α following vaccination in individuals exposed to SARS-CoV-2 reflects proper adaptive immune response including rapid pathogen recognition, prevention of viral replication, and contribution to the long-term defense. However, dramatically increased serum TNF α levels (1000-2500 pg/mL) have been reported in critically ill patients with lung involvement ³50%, regardless of vaccination status. Similarly, Pencheva et al. [53] reported TNF α levels >1000 pg/mL in the most severe cases. In our study, TNF α levels were approximately 1.5-fold higher in vaccinated than in unvaccinated patients. Spearman rank correlation analysis also showed positive correlations between TNF α and IL-1b, IL-1Ra, IL-6, IL-8 and IL-10 in NVP and/or VP groups. TNF α is a well-established activator of NF- κ B. Upon binding to its receptor on the cell surface, TNF α triggers a series of intracellular events leading to NF- κ B activation. This, in turn, induces the transcription of various genes, including those encoding inflammatory cytokines [54].

4.6.

Cytokine storm syndrome has been observed not only in COVID-19 but also in several other disease states associated with immune dysregulation [55], including viral hemorrhagic fevers [56], rheumatoid arthritis [57, 58], inflammatory bowel disease [59, 60], severe sepsis [61, 62]. Cytokine dysregulation also represents a frequent and well-recognized feature of the aging process [63, 64]. In clinical studies, an age of 65 or older is the standard benchmark used to define an aging immune system [25]. In our study, age-dependent changes were already visible in the distribution of immune cells among unvaccinated and vaccinated patients. PIV was higher among NVP aged ³65 years (Median 506) than among NVP aged <65 years (Median 315). In contrast, VP showed an opposite age-related pattern, with lower PIV values among older (Median 658) than younger patients (Median 1090). This confirms that age strongly shapes vaccine responses by altering the numbers, distribution, and functional states of immune cells [65]. Patients aged ³65 years had higher levels of IL-1b, IL-1Ra, IL-6, IL-8, IL-10, TNF α and INF γ compared to patients aged <65 years, irrespective of vaccination status. The most pronounced age-related differences were recorded for pro-inflammatory cytokines IL-1b, IL-6 and TNF α (p <0.001). Older patients also had a higher IL-6/INF γ ratio (Median 1.25) than younger patients (Median 0.59), which indicates an uncontrolled inflammatory response (driven by IL-6) and simultaneously an ineffective antiviral response (indicated by low INF γ) [66]. Our previous meta-analysis demonstrated elevated IL-6 levels in older adults across different disease entities. The reported odds ratio (OR) indicated a potential role of IL-6 in the progression of age-related comorbidities (OR: 1.03, 95%CI 1.01-1.05, p =0.0029) [67]. Age-related comorbidities, including cardiovascular disease (CVD) and associated metabolic risk factors, enhance the hyper-inflammatory response, leading to multi-organ failure [68, 69]. In our cohort, more than 50% of older patients had CVD and/or diabetes (Table 1). Previous studies have also demonstrated that the probability of survival decreases considerably faster in older than younger patients during hospitalization [43]. A multicenter retrospective study conducted in Bangladesh revealed a high prevalence of CVD (42.2%) and diabetes (32.7%) among hospitalized patients with COVID-19, with both comorbidities strongly associated with increased mortality risk. Notably, patients with both conditions exhibited a nearly 7-fold higher risk of death compared to those without comorbidities [20, 43, 70]. Strategies including antivirals, immunomodulators, and vaccines are necessary to prevent adverse health outcomes

and optimize immunity in older adults. In general, post-vaccination increases in cytokines, including IL-1b, IL-6 and TNFa, support the early stages of adaptive immunity, including antibody synthesis and development of long-term cellular memory, thereby preventing clinical complications upon subsequent exposure to SARS-CoV-2 [71, 72]. On the other hand, dangerously high cytokine levels resulting from the overlap of immune processes i.e., post-vaccination immune activation and immunosenescence may compromise the protective effect of vaccination [73]. In this study, all patients aged ≥ 65 years exhibited cytokine storm syndrome and comparable mortality rates, regardless of vaccination status. Hyperinflammation generally reduces vaccine effectiveness; however, paradoxically, some enhanced vaccines (high-dose, adjuvanted) leverage inflammation to improve responses in older adults, showing better immunogenicity than standard vaccines [74-79].

5. Limitations

Caveats of this study include the relatively small sample size. Although immunoenzymatic data are reported for a total of 79 COVID-19 cases here, robust testing will require larger cohorts. Though comorbid medical conditions and other factors play a role in COVID-19 disease severity, this study was not powered to distinguish such factors. Independent immunoinflammatory test sets will also be important in future studies.

6. Conclusions

Identification and quantification of inflammatory biomarkers following vaccination and confirmed SARS-CoV-2 infection are important to provide information on both post-infection and vaccine-induced protective immune responses as well as disease severity. Age strongly shapes the vaccine response by altering the numbers, distribution, and functional states of neutrophils, and complicates the course of COVID-19. The predictive value of neutrophil-related cytokines (IL-1b, IL-6, IL-8 and TNFa) increases when the pan-immune-inflammation value is included in the inflammatory profiling.

Declarations

Conflicts of interest:

4. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contribution

Conceptualization: B.K., A.Z-L.; Methodology: B.K., A.Z-L.; Formal analysis: B.K., M.W.; Investigation: B.K., J.K., M.M., J.Z., E.W-G., B.M.; Writing-original draft: B.K., A.Z-l.; Writing-review and editing: B.K., A.Z-L.; Supervision: A.Z-L. All authors have read and agreed to the published version of the manuscript.

Data Availability

Data availability statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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Figures

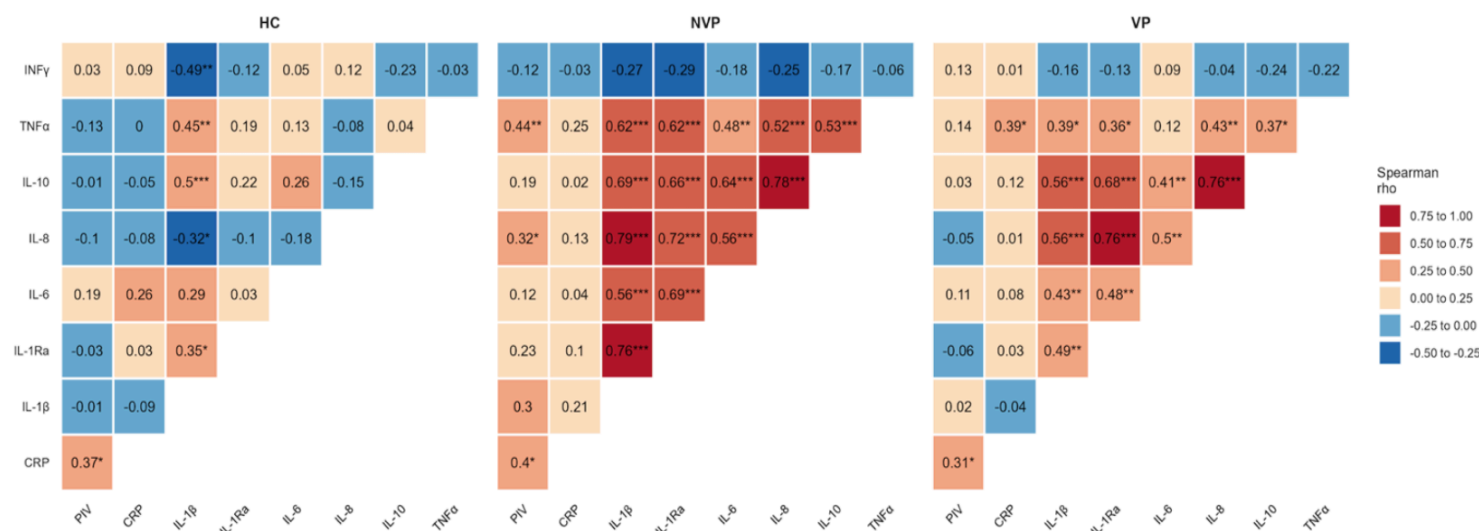


Figure 1

A heatmap illustrating the correlation between different variables. HC healthy unvaccinated comparator, NVP unvaccinated patients with COVID-19, VP vaccinated patients with COVID-19, PIV pan-immune inflammation value, CRP C-reactive protein, IL-1β interleukin 1β, IL-1Ra interleukin-1 receptor antagonist, IL-6 interleukin 6, IL-8 interleukin 8, IL-10 interleukin 10, TNFα tumor necrosis factor α, IFNγ interferon γ. The intensity of color reflects the strength of the correlation (Spearman's rank correlation); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

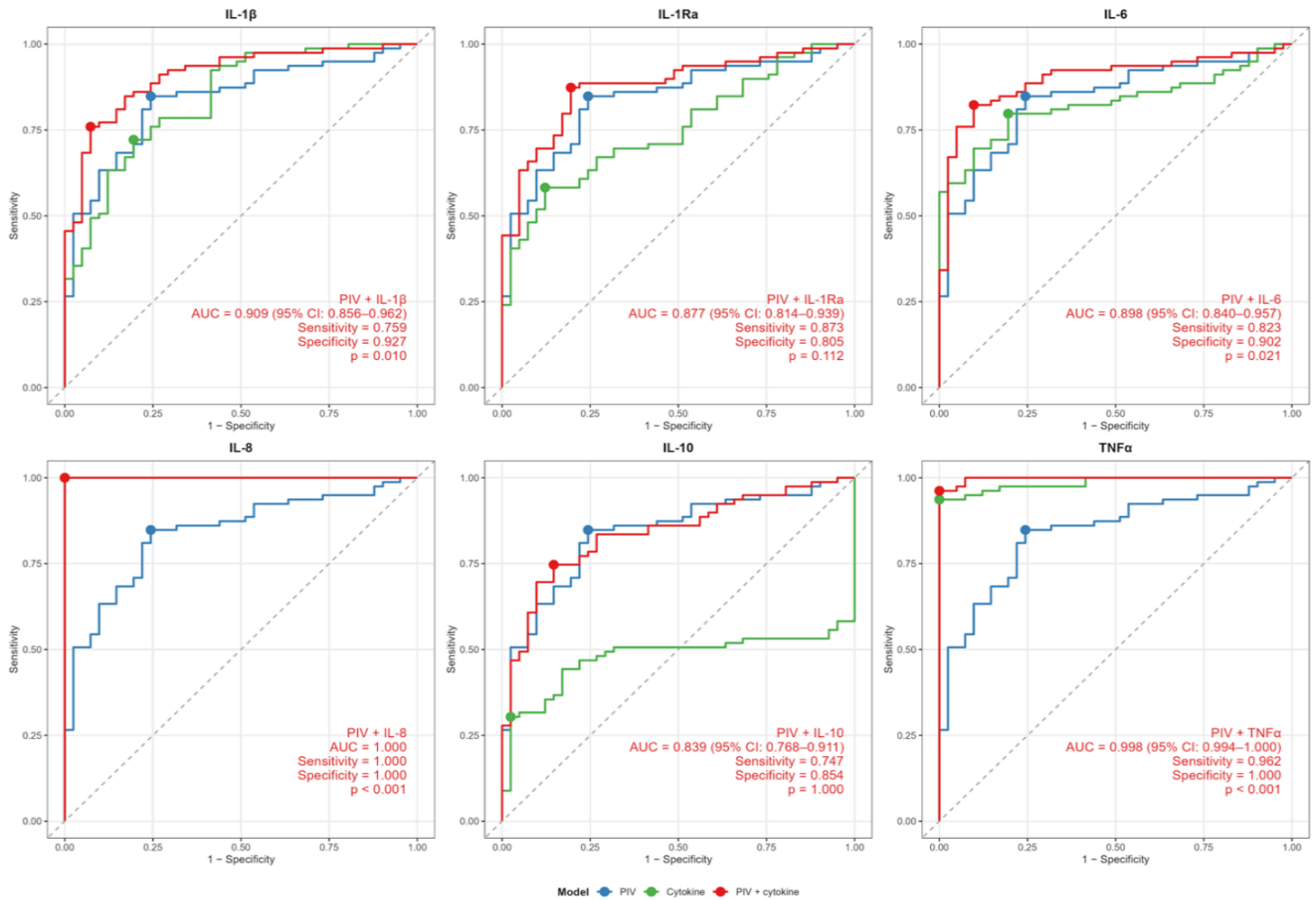


Figure 2

Receiver operating characteristic (ROC) curves for the discrimination of healthy individuals from COVID-19 patients.

Red curves represent multivariable logistic regression models combining PIV (pan-immune-inflammation value) with inflammatory variables. Blue and green curves represent univariate models based on PIV alone and single inflammatory variables i.e., IL-1 β interleukin 1 β , IL-1Ra interleukin-1 receptor antagonist, IL-6 interleukin 6, IL-8 interleukin 8, IL-10 interleukin 10 and TNF α tumor necrosis factor α .